

Stimulatory Beetle Volatiles for the Asian Longhorned Beetle, *Anoplophora glabripennis* (Motschulsky)

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Two male-specific beetle volatiles were found that elicited strong gas chromatographic-electroantennographic responses from both sexes of Asian longhorned beetle adults, *Anoplophora glabripennis*. The secretion consisted of a ~1:1 (v/v) blend of functionalized dialkyl ethers, 4-(*n*-heptyloxy)butanal and 4-(*n*-heptyloxy)butan-1-ol. These compounds are chemically unusual natural products that are previously unknown from insects. Laboratory olfactometer studies showed that a blend of 10 µg of each synthetic compound on a filter paper strip was significantly attractive to ALB adults.

Introduction

Anoplophora glabripennis (Motschulski), the Asian longhorned beetle (ALB), has been inadvertently exported from Asia, probably China, into the United States and Europe via untreated wooden packing material used in international cargo (Milius, 1999). This invasive species was first discovered in New York City in 1996 (Anonymous, 1996), in Chicago in 1998 (Anonymous, 1998), and then in Braunau (upper Austria) in 2001 (Anonymous, 2001).

The ALB belongs to the family Cerambycidae, known as hardwood-borers, in the subfamily Lamiinae whose species usually attack living trees. *A. glabripennis* seems to prefer to attack maple (*Acer*) and horse chestnut (*Aesculus hippocastanum*) in the U.S., and Norway maple (*A. platanooides*) in the Austria, but is also known to infest poplar (*Populus*), willow (*Salix*), elm (*Ulmus*), mulberry (*Morus*), and black locust (*Robinia pseudoacacia*) in its native distribution of eastern China, Japan, and Korea (Anonymous, 1996; Knodel, 1997). Besides maple (*Acer*) and horse chestnut (*Aesculus hippocastanum*) in the United States, a wide variety of other trees may also attract *A. glabripennis*. In the laboratory, *A. glabripennis* will oviposit on cut logs of red oak (*Quercus rubra*) and can survive at least 90 days after implantation as a first instar larva under the bark

of red oak (Hoover *et al.*, personnel communication). *A. glabripennis* has enormous destructive potential because it spends most of its life as a grub inside tree trunks or branches, larval boring in the trunk and large branches inhibit the tree's vascular system and cause severe damage and eventually tree breakage and death (Cavey *et al.*, 1998). So far no natural enemies that effectively control this pest have been identified (Milius, 1999). Soil injection, trunk injection, and bark spray of certain pesticides were found to be effective for ALB control (McLane *et al.*, 2000); however, the utilization of these technologies may be limited to urban forestry. Efforts to control or eradicate this exotic species have led to removal and destruction of all trees showing symptoms of attack in Chicago and New York City (Anonymous, 1998; Haack *et al.*, 1997). These eradication efforts are already costing many millions of dollars annually but, should the ALB escape eradication, estimates of damage to forestry and the nursery industry run well into the billions of dollars.

To date, detection of *A. glabripennis* infestation relies on visual inspection for adult beetles or for signs of their emergence from trees, procedures which are believed to detect at best only ~30% of the infested trees (Milius, 1999). In an attempt to identify and develop an attractant that would be useful for detection and monitoring of this insect, we collected and analyzed airborne volatiles of *A.*

glabripennis. Comparison of gas chromatograms of volatiles trapped from the individual sexes maintained on twigs of a favored host, Norway maple, and from the maple twigs alone, enabled us to distinguish between compounds of insect or plant origin, and whether, in the former case, they were common to both sexes or were sex-specific. Because only limited numbers of beetles were available, with none available for field studies, we relied on gas chromatography coupled with electroantennographic detection (GC-EAD) to focus on compounds eliciting a neural response in the beetle. Although an antennal response is not necessarily a predictor of behavior, it is reasonable to expect that most, if not all, volatile compounds capable of eliciting a behavioral response would also stimulate an antennal response. Indeed, the laboratory bioassays conducted do confirm that the identified compounds are behaviorally active.

Results and Discussion

A. glabripennis volatiles

Two EAD-active compounds, absent from samples derived from females or maple twigs alone, appeared in volatiles from adult male beetles (Fig. 1). Each compound elicited EAD-responses from both male and female beetles (Fig. 2B). No close matches for their EI-MS were retrieved from the Wiley 275 mass spectral database. Molecular weights (mw's) of the two unknowns were determined by chemical ionization mass spectrometry

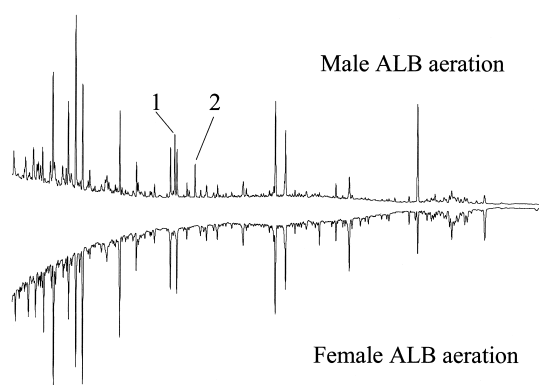


Fig. 1. Gas chromatograms (FID detection) of aeration extracts of male (top) versus female (bottom) Asian longhorned beetle, *A. glabripennis* (groups of 6 beetles aerated 24 hours). Two male-specific compounds are indicated as **1** and **2**.

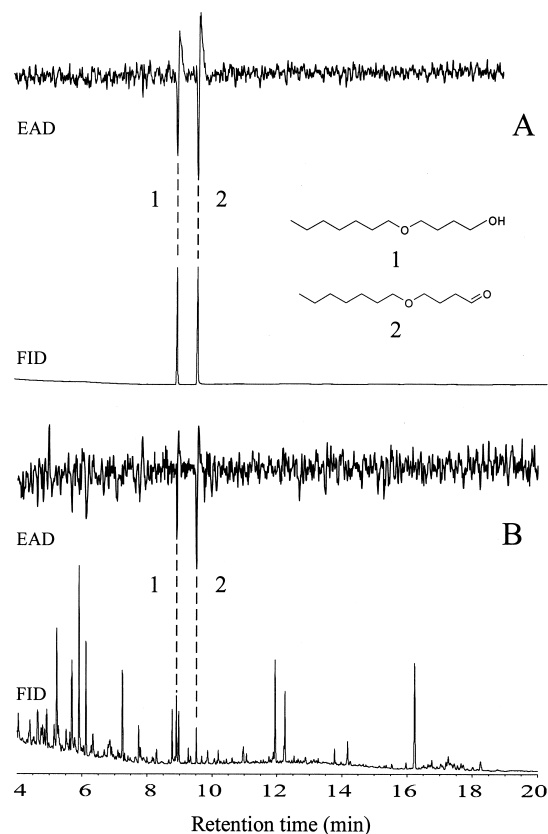


Fig. 2. Simultaneous flame ionization detector (FID) and electroantennographic detection (EAD) responses of an adult male *A. glabripennis* antenna to A) a mixture of synthetic **1** and **2** (50 ng each compound, 1:1 ratio, v/v), and B) volatiles trapped from male *A. glabripennis*.

(CI-MS) obtained with ammonia (NH_3) as reagent gas as mw 186 (m/z 187 $[\text{M}+\text{H}]^+$, m/z 204 $[\text{M}+\text{NH}_4]^+$) and mw 188 (m/z 189 $[\text{M}+\text{H}]^+$, 206 $[\text{M}+\text{NH}_4]^+$), respectively. Comparison of CI-MS obtained with deuterioammonia (ND_3) indicated that the mw 188 compound contained a single exchangeable hydrogen (m/z 191 $[\text{M}-\text{H}+2\text{D}]^+$, m/z 211 $[\text{M}-\text{H}+\text{D}+\text{ND}_4]^+$), whereas in the mw 186 compound no hydrogens were exchanged (m/z 188 $[\text{M}+\text{D}]^+$, m/z 208 $[\text{M}+\text{ND}_4]^+$). The electron ionization MS of the mw 188 compound resembled that of an aliphatic alcohol except for the curious “doubling” of several alkyl fragments (m/z 41/43, 55/57, 71/73) that was observed to resemble a similar phenomenon in the spectrum of 2-(n-butoxy)ethanol and ultimately was explained by the presence of an ether oxygen in the otherwise unsubstituted

chain. 4-(*n*-Heptyloxy)butan-1-ol (**2**) was synthesized and found to be identical (GC on polar and non-polar capillary columns, GC-MS) to the natural product. Swern oxidation (Mancuso *et al.*, 1978) provided the aldehyde **1** which was identical in all respects to the second unknown. Synthetic **1** and **2** each stimulated strong antennal responses from both male and female beetles (Fig. 2A).

Although simple compounds, ethers **1** and **2** appear to be unique as natural products. Aldehyde **1** has appeared once previously in the literature, but only as the product of synthetic methodology (Coutrot and Savignac, 1977). Dialkyl ethers appear to be very uncommon as insect-produced products (Hardie and Minks, 1999; Mori, 1992). The methoxyacetamide of 3-methylbutylamine has been reported from males of two species of fruit flies in the genus *Bactrocera* (Fletcher and Kitching, 1995). Cyclic ethers, particularly cyclic ketals, are relatively common, and a few alkyl aryl ethers have been reported, but they can all be envisioned as products of quite different biosynthetic sequences than those likely to be utilized for dialkyl ether biosynthesis.

During collection of volatiles, compounds **1** and **2** were detectable in all samples from male beetles but were undetectable in samples from female beetles. The concentrations seemed to increase from barely detectable levels in volatiles from newly emerged males to ~30 ng/male/day at about 10 days; older males have not been examined.

Olfactometer bioassay

In the olfactometer bioassay, a dosage study indicated that the lower and upper stimulant thresholds were critical. It was found that more beetles were attracted to the 10 and 20 µg lures than to the lower and higher doses (Fig. 3). Therefore 20 µg of the 1:1 (v/v) blend was chosen for the remaining experiments.

The time required for a beetle to reach the treatment or control chambers varied greatly. Some beetles walked into the treatment chamber in less than two minutes whereas others required as much as twenty minutes to reach a chosen chamber. In total, 69 beetles were tested at the optimized dosage (20 µg of the 1:1 synthetic blend), of which 52% were attracted by the stimulant blend. Of beetles that made a choice, the stim-

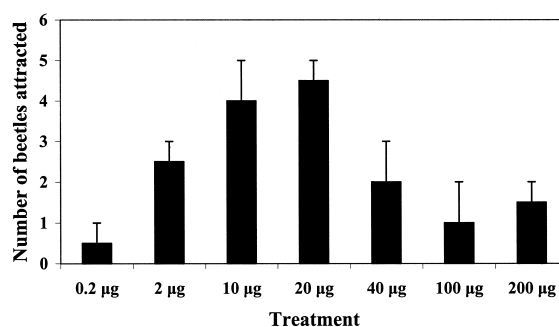


Fig. 3. Attraction of male *A. glabripennis* in an olfactometer baited with synthetic stimulus blend (1:1 ratio, v/v) at seven doses (10 replicates for each dosage).

ulus was significantly preferred over the control chamber, but neither stimulus nor control vs. no-choice beetles was significantly different (Chi-square goodness of fit test: Chi-Square = 13.87, df = 2, *p*-value § 0.0008). No statistical differences in response were detected between different age groups of ALBs, or between female vs. male responses to the synthetic blend.

Experimental

Insects

Virgin *A. glabripennis* adults, collected as larvae or pupae in logs from infestation areas in Chicago and New York City, were maintained in the quarantine facility of the U. S. Department of Agriculture, Otis Plant Protection Center located in Massachusetts. Adults were collected daily upon emergence in April 1999, and separated by sex; most beetles were therefore believed to be unmated, but occasional mating was observed almost immediately upon emergence. Adults were maintained individually in 2-liter plastic bottles containing a water cup and twigs of *A. platanoides* in a rearing room maintained at 16L: 8D, 25 °C, and ~60% relative humidity.

Volatiles collection

Groups of ten 1-day-old male and female *A. glabripennis* were separately introduced into 1-liter, 4-necked glass containers (Zhang *et al.*, 1994) containing freshly cut twigs of *A. platanoides* in a water cup. Air was drawn into the container through 6–14 mesh activated charcoal (Fisher Sci-

entific, Pittsburgh, PA), and out of the container through two traps (15 cm X 1.5-cm OD) containing Super Q (200 mg each; Alltech Associates, Inc., Deerfield, IL) using a water aspirator or vacuum pump (~1 liter/min). Beetles were aerated continuously for 8–10 days, and adsorbents were changed every 24 hours. The adsorbents were eluted with methylene chloride (4 X 0.5 ml); the eluates were concentrated and stored at –4 °C until use. The same system, but without beetles, was used for collection of plant volatiles.

Instrumentation

The coupled GC-EAD system used was as previously described (Zhang *et al.*, 1999; Zhang *et al.*, 1997) with a few modifications. A Hewlett Packard 6890 gas chromatograph equipped with a 60 m X 0.25-mm ID, 0.25- μ m film-thickness DB-5 or DB-WAXetr capillary column (J&W Scientific Inc., Folsom, CA) in the splitless mode with nitrogen as carrier (2 ml/min) was used for GC-EAD analysis (50 °C for 2 min, then programmed to 230 °C at 10 °C/min and held for 10 min). An acrylic well type holder connected to a high-impedance 1:100 amplifier with automatic baseline drift compensation was employed, and the antennal preparation was cooled to ~5 °C inside a condenser by circulating near 0 °C water from a benchtop refrigeration unit (RTE-100, NESLAB instruments, Inc., Portsmouth, NH). The flame ionization and electrophysiological output signals were recorded using Hewlett-Packard ChemStation software. Identical units were assembled and used in the Chemicals Affecting Insect Behavior Laboratory, Beltsville, MD, where some of the work using male beetles was done, and in the Otis Plant Protection Center quarantine facility, MA, where experiments with both sexes were conducted.

Gas chromatography-mass spectrometry (GC-MS) was conducted on a Hewlett-Packard 6890 coupled to a HP 5973 Mass Selective Detector (EI) using an identical GC column and conditions but with helium as carrier. Chemical ionization (CI) spectra were obtained from a Finnigan Model 4510 GC-MS with ammonia or deuterioammonia as reagent gases.

Chemicals

4-(*n*-Heptyloxy)butan-1-ol (**2**) was prepared by Williamson synthesis. A solution of butane-1,4-diol (180 g, 2 mol) in dry *N,N*-dimethylformamide (1 liter) was stirred under a nitrogen atmosphere and cooled with an ice bath while sodium hydride (60% in mineral oil, 76 g, 1.9 mol) was added in portions over about 10 minutes. The solution was slowly heated to 60 °C, then again cooled and a solution of 1-bromoheptane (190 ml, 1.21 mol) in dry tetrahydrofuran (200 ml) was added dropwise. After the addition, the solution was again heated to 60–70 °C, then was cooled and added to ice (1 kg). The mixture was extracted with ether-hexane (1:1, 3 X 200 ml), and the combined extracts were rinsed with water (2 X 200 ml) and finally with saturated sodium chloride solution (1 X 200 ml). After drying over magnesium sulfate, the solvent was removed with a rotary evaporator and the residue was distilled to give 156.6 g (69%) of **2**, b.p. 88–91 °C, 0.1 Torr. ¹H NMR (CDCl₃, 300 MHz) 0.866 (3 H, distorted t, *J* = 7.0 Hz, CH₃), 1.27 (methylene), 1.51–1.6 (2H, m), 1.63–1.69 (4 H, m), 2.60 (br. s, OH), 3.41 (2H, t, *J* = 6.75 Hz, CH₂OCH₂), 3.44 (2H, t, *J* = 5.75 Hz, CH₂OCH₂), 3.63 (2H, t, *J* = 5.25 Hz, CH₂OH); EI-MS *m/z* 145 (5), 115 (16), 98 (10), 97 (25), 89 (44), 73 (67), 71 (97), 70 (15), 69 (12), 57 (100), 56 (25), 55 (99), 44 (20), 43 (72), 42 (29), 41 (62).

4-(*n*-Heptyloxy)butanal (**1**) was obtained by Swern oxidation of (**2**). A solution of oxalyl chloride (42 ml) in dry methylene chloride (750 ml) was cooled under a nitrogen atmosphere to –75 °C. Dimethylsulfoxide (72 ml) was added dropwise with stirring, followed, after ten minutes by a solution of **2** (75.2 g) in methylene chloride (75 ml). The mixture was stirred and allowed to warm to –45 °C, then was again cooled to –75 °C and treated dropwise with triethylamine (292 ml). After the addition was complete, the cooling bath was removed and the mixture was allowed to slowly warm to room temperature and stir one hour. Ice was added, and the product was partitioned between water and methylene chloride. The solvent was removed with a rotary evaporator, and the residue was dissolved in petroleum ether (500 ml) and the solution was rinsed successively with water, cold aqueous hydrochloric acid, water, and saturated aqueous sodium bicarbonate. After

drying over magnesium sulfate, the solvent was stripped *in vacuo*, and the residue was distilled to give 57.6 g (77%) of **1**, b.p. 60 °C, 0.25 Torr. ¹H NMR (CDCl₃, 300 MHz) 0.869 (3 H, distorted t, *J* = 7.0 Hz), 1.27 (methylene), 1.53 (2 H, m), 1.90 (2 H, m), 2.51 (2 H, td, *J* = 7.0 and 1.5 Hz), 3.37 (2 H, t, *J* = 6.5 Hz, CH₂OCH₂), 3.42 (2 H, *J* = 6.25 Hz, CH₂OCH₂), 9.77 (1 H, t, *J* = 1.5 Hz, CHO); EI-MS *m/z* 142 (23), 127 (13), 98 (17), 97 (19), 96 (26), 71 (58), 70 (28), 69 (19), 57 (100), 56 (24), 55 (30), 44 (14), 43 (76), 42 (29), 41 (71).

Olfactometer and bioassay

A typical two-choice olfactometer was used to test biological activity of synthetic samples. Three round-bottom flasks (1 liter, 24/40) were attached to each end of the glass Y-tube (300 mm X 70-mm OD) with glass joints (71/60) as the sample, treatment, and control chambers. Treatment and control chamber connectors were connected to a T-tube using silicon tubing (5.0 mm ID, 9.5 mm OD, Silastic® laboratory tubing, VWR Scientific Products Corp., San Francisco, CA) and the end of this T-tube was then connected to a water bubbler and charcoal trap (Activated carbon, 6–14 mesh, Fisher Scientific, Pittsburgh, PA; 40 mm X 20 mm-OD). The release chamber was connected to a vacuum pump through a flow meter (Accura Flow Products Inc., Hatboro, PA) adjusted to a flow rate at ~1 liter/min. Two identical apparatuses were positioned horizontally on a table in the center of the room (22–23 °C, ~60% relative humidity) with three incandescent lights (40W/each) directly above the olfactometers ~1.5 meters.

A dosage study was conducted in order get the lower and upper stimulant thresholds. The filter paper strips were baited with 0.2, 2, 10, 20, 40, 100, and 200 µg of a 1:1 blend of the synthetic male-specific compounds, and each dosage was tested two times on two different days with a group of five males. A total of ten replicates were performed using each dosage and a blank as a control.

At the optimized dose, beetles of various ages were tested; the youngest was 1-day-old (newly emerged) and the oldest was 51-day-old. Both males and females were used as available. A total of 69 replicates (17 males and 52 females) were performed using 1 beetle per replicate to prevent

fighting between males or attempting to mate between males and females. Twenty µg of synthetic male-specific stimulus **1** and **2** (1 µl hexane solution, 10 µg/µl each, 1:1 ratio) were loaded on a filter paper strip, which was placed into the treatment chamber connector. As a control, the same volume of hexane on a filter paper strip was placed into the control chamber connector. Beetles were removed from their rearing bottles and released into the end of the Y-tube (release chamber side) and observed for a period of 20 min; beetles that did not complete the entire upwind walking choice within twenty minutes, or did not move at all, were counted as “no choice”. After each assay, the beetle was removed and a new beetle was introduced. Treatment and control chambers were alternated every two assays to randomize uneven environmental factors.

Statistical analysis

The *A. glabripennis* responses were analyzed using StatXact 4 (Cytel Software) to calculate exact *p*-values for the choices (Metha and Patel, 2000). All beetles' responses were analyzed in three ways: choice, choice by sex, and choice by age. When overall statistical significance was found, Chi-square tests were used to compare pairs of cells to determine if they were different. Sidak adjusted *p*-values were used for the comparisons so that the overall significance level was 0.05.

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